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Identification of Alpha-Hemolytic Streptococci by Pyrosequencing the 16S rRNA Gene and by Use of VITEK 2[▽]

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Alpha-hemolytic streptococci are very difficult to identify by phenotypic methods. In this study, a pyrosequencing method for the identification of streptococcal species based on two variable regions of the 16S rRNA gene is described. Almost all studied streptococcal species ($n = 51$) represented by their type strains could be differentiated except for some closely related species of the *Streptococcus bovis* or *S. salivarius* group. The pyrosequencing results of alpha-hemolytic streptococci isolated from blood ($n = 99$) or from the normal pharyngeal microbiota ($n = 25$) were compared to the results obtained by the VITEK 2 with GP card (bioMérieux, Marcy l'Etoile, France). As expected, the results of the two methods did not completely agree, but 93 (75.0%) of the isolates assigned to the same streptococcal group by both methods and 57 (46.0%) reached consistent results at the species level. However, 10 strains remained unidentified by VITEK 2, and 4 isolates could not be assigned to any streptococcal group by pyrosequencing. Identification of members of the *S. mitis* and *S. sanguinis* groups proved difficult for both methods. Furthermore, the pyrosequencing analysis revealed great sequence variation, since only 43 (32.3%) of the 133 isolates analyzed by pyrosequencing had sequences identical to a type strain. The variation was greatest in the pharyngeal isolates, slightly lower in the blood culture isolates, and nonexistent in invasive pneumococcal isolates ($n = 17$) that all had the *S. pneumoniae* type strain sequence. The resolution of the results obtained by the two methods is impeded by the lack of a proper gold standard.

The genus *Streptococcus* comprises a heterogeneous group of bacteria, some of which are major pathogens and some are members of the normal microbiota of humans and animals. Identification of streptococcal species has traditionally been based on hemolysis on blood agar, biochemical tests, and serological grouping. During the last decade, numerous molecular methods, such as ribotyping (25), amplified ribosomal restriction analysis (32), PCR-based methodology (10), oligonucleotide probes (1, 20), and sequencing of different targets (5, 6, 22, 23, 36, 37) have been developed to identify streptococcal species more rapidly and accurately.

Pyrosequencing is a sequencing by synthesis method, in which incorporation of nucleotides is enzymatically transformed to release light which is detected and presented as a peak histogram, a pyrogram (26, 28). The main advantage of pyrosequencing is its rapidity and lower price compared to conventional sequencing. Furthermore, since the dispensation order can be designed individually for each sample, verification of suspected mutations is simple. Also, heterogeneous sequences are quite easily determined, if the possible sequence combinations are known (11). By pyrosequencing fairly short sequences only can be determined, but usually the sequences of around 60 bases obtainable with suitable dispensation orders are sufficient for carefully designed applications. In the field of microbiology, pyrosequencing has already been applied to the identification of bacteria (18, 21, 27) and heterogeneous 23S rRNA gene sequences conferring antibiotic resistance (11, 34), for example. Pyrosequencing of two regions of the RNase P RNA gene, *rnpB*, has been proposed for the identification of streptococci (15).

Sequencing of the 16S rRNA gene is widely used in the identification of bacteria. Although the discriminative power of the 16S rRNA has recently been argued to be to too low for the identification of streptococci and other closely related bacteria (14, 15), it has been found effective in identification of clinically relevant cocci including viridans streptococci (3, 13). Determining the whole 16S rRNA gene sequence may not be necessary since the variable regions contain most of the variation between bacterial species. Indeed, the majority of the variation between 16S rRNA gene sequences of streptococcal species is found in the 5′ terminal part of the gene (13), especially in the regions used in the present study: the v1 and v2 region corresponding to bases 63 to 92 and 193 to 222 of the *S. pneumoniae* R6 16S rRNA (NC_003098), respectively. In our previous study, the v1 region of alpha-hemolytic commensal streptococcal isolates was determined by pyrosequencing and compared to the sequences of selected type strains. Although the variation in the v1 region was extensive, some of the studied type strains had identical v1 sequences (33) and, consequently, analysis of the v2 region was added to the pyrosequencing assay.

VITEK 2 is an instrument for automated phenotypic identification of medically relevant bacteria and yeasts (bioMérieux, Marcy l'Etoile, France). The new colorimetric GP card for identification of gram-positive bacteria consists of 43 biochemical tests that are monitored up to 8 h. The database contains

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33 streptococcal species including two "slashline species": *S. mitis-S. oralis* and *S. lutetiensis-S. bovis*, six subspecies belonging to *S. constellatus*, *S. dysgalactiae*, or *S. equi* and *S. suis* biotypes I and II. Previous studies with VITEK 2 have included only small numbers of viridans streptococci (9, 15). The purpose of the present study was to compare pyrosequencing of the v1 and the v2 regions of the 16S rRNA gene and the VITEK 2 with a colorimetric GP card for identification of alpha-hemolytic streptococci.

MATERIALS AND METHODS

Bacterial strains. Nonpneumococcal alpha-hemolytic blood culture isolates (n = 102) collected in Turku University Hospital derived from 96 patients between 1993 and 2004 were analyzed. Thirty-six strains were single isolations from immunocompromised patients, while sixty-six were from patients with two to eight blood cultures growing alpha-hemolytic streptococci. Eighty-four of the strains were from blood cultures growing only streptococci, and eighteen were from blood with multibacterial growth. Multiple isolates from a single patient were only included if they had been originally identified as separate species, if they had different antimicrobial susceptibility profiles, or if the interval between their isolation was at least 2 weeks. The strains had been originally identified on the basis of alpha-hemolysis and resistance to optochin or using either API 20Strep or VITEK 2 with a fluorogenic identification card (both from bioMérieux) and were stored at −70° until the present study. For comparison, 39 alpha-hemolytic isolates from the normal pharyngeal microbiota of six elderly persons (33) and 17 invasive *S. pneumoniae* clinical isolates collected in Finland were included. The *S. pneumoniae* isolates had typical colony morphology and hemolysis, and they were optochin susceptible. The isolates were grown on blood agar and incubated at 35°C in an atmosphere of 5% CO_2. Three blood culture isolates were identified as *Enterococcus*, *Granulicatella*, or *Lactobacillus*, respectively, based on the pyrosequencing results and database searches. By VITEK 2, these isolates were identified as *Enterococcus avium*, *Gemella morbillorum*, and an unidentified organism, respectively. Similarly, one *Granulicatella* and four *Enterococcus* isolates were found among the pharyngeal isolates. Unfortunately, only 25 pharyngeal streptococcal isolates were viable for the VITEK analysis. Consequently, 99 blood culture isolates and 25 pharyngeal isolates were analyzed by both methods, and 9 pharyngeal isolates were analyzed only by pyrosequencing.

Streptococcal type strains (n = 59) and three additional well-characterized strains (Table 1) were cultured according to the recommendations of the bacterial strain depositors. In addition to the subspecies listed in Table 1, the type strains of *S. bovis*, *S. caprinus* and *S. infantarius* subsp. *coli* were considered as subspecies of *S. equinus*, *S. gallolyticus* subsp. *gallolyticus* and *S. lutetiensis*, respectively (24, 31, 35). Consequently, 51 species were represented by the 59 type strains listed in Table 1. In addition to the species listed in Table 1, the 16S rRNA gene sequences of *S. castoreus*, *S. equi* subsp. *ruminatorum*, *S. gallinaceus*, *S. oligofermentans*, and *S. pseudopneumoniae* type strains were retrieved from the GenBank and added to the local sequence database.

Template preparation and PCR amplification. The PCR templates were prepared by dissolving bacterial colonies in water and inactivating the solution at 95°C for 10 min. The biotinylated PCR product covering the v1 and v2 regions was generated using primers BioStrepV1For (5′-Bio-AGTTTGATCCTGGCTC AGGACG-3′) and StrepV2Rev (5′-CAACTAGCTAATACAACGCAGGTC-3′). The 50- to 100-μl PCRs contained 0.3 μM concentrations of primers, 0.015 U of AmpliTaqGold DNA polymerase/μl, 1× Geneamp PCR Gold buffer, 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.2 mM concentrations of deoxynucleoside triphosphates (Amersham Biosciences, Piscataway, NJ), and template at 1/10 of the reaction volume. Thermal cycling consisting of initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 15 s at 57°C, and 30 s at 72°C was performed by using a PTC-200 thermal cycler (MJ Research, Waltham, MA). The presence of the approximately 250-bp PCR product was verified on an agarose gel.

Pyrosequencing. Pyrosequencing was performed by using streptavidin-coated Sepharose beads (Amersham Biosciences), an PSQ 96 MA instrument, a vacuum prep workstation, and PyroGold SQA reagents (Biotage AB, Uppsala, Sweden) according to the instructions of the manufacturer. A portion (20 μl) of the PCR product and 15 pmol of the sequencing primer StrepV1RevV2 (5′-CCTACGC GTTACTCACCCGTTC-3′) or StrepV2RevV2 (5′-ACTAGCTAATACAACG CAGGTCCA-3′) was used in each sequencing reaction. To save reagents, 6 μl of enzymes and substrates were added manually to the reaction wells. The dispensation order for the v1 region was GCGACTC10(ACTG), and for the v2 region it was TCTACAGTCGA10(CGTA). The sequences obtained by pyrosequencing were handled by using a BioEdit sequence editor (12). Thereafter, 30-base v1 and v2 sequences were concatenated and compared to the corresponding sequences of type strains by using the Identifire 1.0.5.0 software (Biotage). The software gave the isolates a score which was 100 if the sequences of the isolate and a type strain were identical. One mismatch in the 60-base sequences generated a score of 96.9, and a score of ≥96.9 was considered an acceptable identification.

Partial 16S rRNA gene sequencing. To verify some of the pyrosequencing results distinct from the type strains, approximately 1,000 bp of the 16S rRNA gene of nine pharyngeal isolates (Table 3) was sequenced. The PCR primers fd1mod2 (5′-AGAGTTTGATCMTGGCTCAG-3′) and rp2 and the sequencing primers 357f, 357r, 533f, 533r, and 907r (17) were used. For the *S. acidominimus*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. pluranimalium*, *S. entericus*, *S. equi* subsp. *zooepidemicus*, *S. hyovaginalis*, and *S. iniae* type strains a sequence identical to the sequences determined by pyrosequencing could not be found in the GenBank, and their 5′ terminal part of the 16S rRNA gene was determined using sequencing primer 533r. The PCR products were purified with the High-Pure PCR product purification kit (F. Hoffmann-La Roche, Ltd., Basel, Switzerland) and sequenced by using the ABI Prism BigDye Terminator v3.0 (Applied Biosystems). The sequences were processed with Vector NTI advance 10.1.1 software (Invitrogen Corp., Carlsbad, CA), and the pharyngeal sequences were subjected to an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were in agreement with the ones obtained by pyrosequencing, good correlation between the pyrosequencing result and the BLAST search result was found, and even heterogeneous peaks could be detected at the positions that agreed with the pyrosequencing results.

VITEK 2 analysis. The streptococcal type strains and alpha-hemolytic isolates were analyzed by the VITEK 2 system using the GP card and WSVT2-R04.01 software (bioMérieux) according to the instructions of the manufacturer. The results were interpreted so that the isolates belonging to a species not included in the VITEK database were satisfactorily identified if the result belonged to the same streptococcal group with the known species or the pyrosequencing result. If VITEK 2 analysis resulted in low discrimination between two species and an additional Voges-Proskauer test proposed by the device resulted in the same species or group with the pyrosequencing result, the results were regarded as concordant. VITEK 2 proposed some other additional tests for the resolution of "low-discrimination" results, but unfortunately they were not available in our laboratory, and thus such results remained unresolved.

RESULTS

Identification of streptococcal type strains. When the 30-base v1 and v2 sequences were combined, 46 of the 51 studied species could be differentiated, and two sequence combinations were shared by two or more species (Table 1). The first was found in the *S. bovis* group members *S. bovis* and *S. lutetiensis*, and the second in the *S. salivarius* group members *S. salivarius*, *S. thermophilus*, and *S. vestibularis.* However, 53 different sequence combinations were found among the 59 studied type strains since some studied subspecies also contained slightly different sequences (Table 1). For example, the sequence of the *S. equinus* type strain differed from the sequence of the *S. bovis* type strain by one nucleotide, revealing small intraspecies variation. Small sequence differences were found also between the different *S. gallolyticus* subspecies. However, the sequences of the analyzed *S. pneumoniae* and *S. pyogenes* reference strains were identical to the sequences of the type strains (Table 1), and all of the different alleles of the streptococcal genomic sequences available in the GenBank were identical to their respective type strains in the studied regions. The smallest difference between distinguishable type strains in the combined v1 and v2 region was one nucleotide, which was detected between three species pairs of the *S. bovis* group, whereas the smallest difference between species of the *S. mitis* group was two nucleotides. Except for seven type strains, a type

TABLE 1. Streptococcal type or reference strains analyzed in this study[a]

Strain[b]	Pyrosequencing		VITEK result	
	v1+v2 sequence[c]	GenBank no.[d]	Species	Confidence level[e]
Beta-hemolytic streptococci				
S. agalactiae[f] LMG 14694^T	1	DQ303183	*S. agalactiae*	VG
S. canis[f] LMG 15890^T	2	AB002483	*S. canis*	VG
S. didelphis CCUG 45419^T	3	DQ303185	*S. dysgalactiae* subsp. *equisimilis*	Acc
S dysgalactiae subsp. *dysgalactiae*[f] LMG 15885^T	4	EF151154	*S. dysgalactiae* subsp. *dysgalactiae* or *S. dysgalactiae* subsp. *equisimilis*	LD
S. dysgalactiae subsp. *equisimilis*[f] CCUG 36637^T	5	AB008926	*S. dysgalactiae* subsp. *equisimilis*	Exc
S. equi subsp. *equi*[f] LMG 15886^T	6	DQ303186	*S. equi* subsp. *equi*	VG
S. equi subsp. *zooepidemicus*[f] LMG 16030^T	7	EF151157	*S. equi* subsp. *zooepidemicus*	Exc
S. iniae CCUG 27303^T	8	EF151159	*S. porcinus*	VG
S. phocae LMG 16735^T	9	AJ621053	*S. dysgalactiae* subsp. *equisimilis*	Acc
S. porcinus[f] LMG 15980 ^{T}	10	AB002523	*S. porcinus*	VG
S. pyogenes[f] ATCC 700294, CCUG 4207^T	11	NC_007297	*S. pyogenes*	Exc
Non-beta-hemolytic streptococci				
S. pneumoniae[f] ATCC 49619, DSM 11867, LMG 14545^T	12	AY281082	*S. pneumoniae*	VG
S. suis[f] CCUG 7984^T	13	AB002525	*S. suis* I or *S. suis* II	LD
S. bovis group				
S. bovis[h] LMG 8518^T	14	AB002482	*S. lutetiensis*/*S. bovis*	Exc
S. equinus[f] LMG 14897^T	15	AF104116	*S. equinus*	Exc
S. gallolyticus subsp. *gallolyticus*[g] CCUG 35224^T	16	AF104114	*S. gallolyticus*	Exc
S. caprinus[g] LMG 15572^T	16	AF104114	***S. gallolyticus*** or *S. hyointestinalis*	LD
S. gallolyticus subsp. *macedonicus*[g] LMG 18488^T	17	Z94012	*S. lutetiensis*/*S. bovis*	VG
S. gallolyticus subsp. *pasteurianus*[g] CCUG 46150^T	18	AJ297216	*S. pasteurianus*	Exc
S. infantarius subsp. *coli* CCUG 47831^T	14	AF429763	*S. pasteurianus*	Good
S. infantarius subsp. *infantarius*[f] CCUG 43820^T	19	AF429762	*S. infantarius*	Exc
S. lutetiensis[h,i] CCUG 46149^T	14	AF429763	*S. infantarius* subsp. *coli*	VG
Viridans streptococci				
S. mutans group				
S. criceti LMG 14508^T	20	AJ420198	*S. mutans*	Exc
S. downei[f] CCUG 24890^T	21	AY188350	*S. sobrinus*	Exc
S. ferus LMG 16520^T	22	AY058218	*S. mutans*	Acc
S. hyovaginalis LMG 14710^T	23	EF151158	*S. uberis*	Acc
S. macacae LMG 15097^T	24	AY188351	*S. alactolyticus*	Acc
S. mutans[f] CCUG 17824^T	25	NC_004350	*S. mutans*	Exc
S. ovis[f,j] LMG 19174^T	26	Y17358	*S. ovis*	Exc
S. ratti CCUG 27642^T	27	AJ420201	*S. thoraltensis* or *S. mutans*	LD
S. sobrinus[f] CCUG 25735^T	28	AY188349	*S. sobrinus*	Exc
S. salivarius group				
S. alactolyticus[f] CCUG 27297^T	29	AF201899	*S. alactolyticus*	VG
S. hyointestinalis[f] LMG 14579^T	30	AF201898	*S. hyointestinalis*	VG
S. salivarius[f] DSM 20560^T	31	AY188352	*S. equinus*	Exc
S. thermophilus[f] CCUG 21957^T	31	NC_006448	ND	-
S. vestibularis[f] DSM 5636^T	31	AY188353	*S. vestibularis*	VG
S. anginosus group				
S. anginosus[f] DSM 20563^T	32	AF104678	*S. anginosus*	VG
S. constellatus subsp. *constellatus*[f] DSM 20575^T	33	AF104676	*S. constellatus* subsp. *constellatus*	Exc
S. constellatus subsp. *pharyngis*[f] CCUG 46377^T	33	AY309095	*S. constellatus* subsp. *pharyngis*	Exc
S. intermedius[f] DSM 20573^T	34	AF104671	*S. intermedius*	Exc
S. sinensis[j] CCUG #0059#^{T}	35	AF432856	*S. constellatus* subsp. *pharyngis* or *S. anginosus*	LD
S. sanguinis group				
S. gordonii[f] DSM 6777^T	36	AY485606	*S. gordonii*	Exc
S. sanguinis[f] DSM 20567^T	37	AF003928	*S. sanguinis* or *S. cristatus*	LD
S. parasanguinis[f] DSM 6778^T	38	DQ303191	*S. parasanguinis* or *S. suis* II	LD
S. mitis group				
S. australis[j] CCUG 45919^T	39	AY485604	*S. mitis* or *S. oralis*	VG
S. cristatus[f] DSM 8249^T	40	AY584476	*S. cristatus*	Exc
S. entericus[j] CCUG 44616^T	41	EF151156	*S. salivarius*	Exc
S. infantis LMG 18720^T	42	AB008315	*S. mitis* or *S. oralis*	Exc

Continued on following page

TABLE 1—*Continued*

Strain[b]	Pyrosequencing		VITEK result	
	v1+v2 sequence[c]	GenBank no.[d]	Species	Confidence level[e]
S. mitis[h] DSM 12643^T	43	AF003929	*S. mitis* or *S. oralis*	Exc
S. oralis[h] DSM 20627^T	44	AY485602	*S. mitis* or *S. oralis*	VG
S. orisratti CCUG 43577^T	45	AF124350	*S. hyointestinalis*	Acc
S. peroris LMG 18719^T	46	AB008314	*S. mitis* or *S. oralis*	VG
Unusual *streptococcus* species				
S. acidominimus LMG 17755^T	47	EF151153	unidentified organism	
S. parauberis LMG 12174^T	48	AF284579	*S. thoraltensis*	Exc
S. *pleomorphus*[i] CCUG 11733^T	49	M23730	ND	
S. pluranimalium[f] LMG 14177^T	50	EF151155	*Granulicatella adiacens*	VG
S. thoraltensis[f] LMG 13593^T	51	Y09007	*S. thoraltensis*	Exc
S. uberis[f] LMG 9465^T	52	AB023573	*S. thoraltensis*	Acc
S. urinalis LMG 19649^T	53	AJ131965	*S. dysgalactiae* subsp. *equisimilis*	Acc

[a] ATCC, American Type Culture Collection, Manassas, VA; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen GmbH, Braunschweig, Germany; LMG, Laboratorium voor Mikrobiologie, Universiteit Ghent, Ghent, Belgium. ND, not determined.
[b] The species are grouped based on the review of Facklam (8). Only the type strains were analyzed by the VITEK 2.
[c] The different sequences of the combined 30-bp v1 and v2 region are indicated by numbers: species with identical sequences have the same sequence numbers.
[d] The GenBank accession number from which the correct v1 and v2 sequences can be found.
[e] The confidence level is a measure of the strength of the VITEK analysis in the descending order Exc (excellent), VG (very good), good, Acc (acceptable), and LD (low discrimination). If VITEK 2 analysis resulted in low discrimination between two species and an additional Voges-Proskauer test proposed by the device could discriminate the species, the final result is in boldface.
[f] Species included in the VITEK GP database.
[g] *S. gallolyticus* is included in the VITEK GP database, but its subspecies are not differentiated except for *S. gallolyticus* subsp. *pasteurianus*, which is identified as *S. pasteurianus*.
[h] *S. bovis* and *S. lutetiensis*, as well as *S. mitis* and *S. oralis*, are identified as "slashline taxa" by the VITEK GP card.
[i] Not grouped in the review of Facklam (8).
[j] Grouping uncertain based on the review of Facklam (8).

strain sequence containing the v1 and v2 sequences identical to the ones obtained by pyrosequencing could be found in GenBank. However, careful searching was required to find the accurate one among the many sequence versions available for many streptococcal type strains. The GenBank entries containing the sequences identical to the pyrosequencing results are presented in Table 2.

With the VITEK 2 system, 30 (81.1%) of the 37 type strains represented in the instrument's database were correctly identified to the species level. In three additional strains the correct result was among two possibilities between which VITEK 2 was unable to discriminate. Twenty-one type strains represented a species not included in the VITEK database. Sixteen of these were members of viridans groups, and thirteen (81.3%) were designated to the correct group (Table 1). The misidentifications mainly concerned uncommon species, but the *S. salivarius* type strain was repeatedly identified as *S. equinus*.

Identification of alpha-hemolytic streptococcal blood culture isolates. The invasive *S. pneumoniae* ($n = 17$) isolates contained the *S. pneumoniae*-specific sequences in both studied regions, and their species designation got verification. In contrast, only 39 (39.4%) of the 99 blood culture isolates had a sequence identical to some of the streptococcal type strains and could be unambiguously assigned to a species by pyrosequencing (Table 2). The most prevalent species were *S. anginosus* ($n = 7$), *S. sanguinis* ($n = 7$), *S. intermedius* ($n = 6$), and *S. salivarius* or *S. vestibularis* ($n = 5$). Thirty (79.5%) of these isolates were identified as the same species with VITEK 2, and thirty-four (87.2%) were identified at least to the same streptococcal group by VITEK 2. Notably, there were no group-level discrepancies in this group of strains. However, four (10.3%) of these isolates could not be conclusively identified by VITEK 2.

Fifty-four (54.5%) blood culture isolates could be designated to the most probable streptococcal species by pyrosequencing (Table 2), but often the result was not clear since the sequence differences between the closest and the second closest type strain were minimal. This was discernible especially with isolates with sequences closest to that of a member of the *S. mitis* group. For example, the most prevalent v1 and v2 sequence combination ($n = 24$) among the blood culture isolates was a sequence differing by one nucleotide from the type strain sequences of *S. mitis*, *S. pseudopneumoniae*, and *S. pneumoniae.* Interestingly, this sequence was found in *S. sanguinis* ATCC 49296 (AY281086). By the VITEK 2 system, 19 (79.2%) of these isolates were identified as *S. mitis*/*S. oralis* with at least a good confidence level, 1 was identified as *S. mitis*/*S. oralis* or a third species with low discrimination, and 4 isolates could not be identified (Table 2). Thus, these isolates most likely belong to the *S. mitis* group. In the *S. sanguinis* group, nine strains with one difference at the maximum to the *S. sanguinis* type strain sequence were identified as *S. sanguinis* also by VITEK 2, and the nine strains with lower sequence similarities were identified as *S. mitis*/*S. oralis* by VITEK 2. The latter ones support the combining of the *S. mitis* and *S. sanguinis* groups (30).

In addition, pyrograms indicating heterogeneous 16S rRNA alleles were detected in six blood culture isolates (Table 2 and Fig. 1). The other region was readable in five of them, and they could be tentatively identified using it. The results of one region were generally concordant with the VITEK results. One isolate identified as *S. mitis*/*S. oralis* by the VITEK had heterogeneous sequences in both regions, but it could, however, be

TABLE 2. Pyrosequencing and VITEK 2 results of the alpha-hemolytic blood culture isolates

Group and no.	Pyrosequencing result[a]		VITEK 2 result		Unresolved (*n*)[d]
	Species	Score[b]	Species	Confidence level[c] (*n*)	
S. anginosus group					
7	*S. anginosus*	100	*S. anginosus*	Exc (4)	
			S. anginosus or *S. gordonii*	LD (2)	
			Inconclusive identification	— (1)	1
3	*S. anginosus*	96.9	*S. anginosus*	Exc (1)	
			S. anginosus or *S. gordonii*	LD (2)	
6	*S. intermedius*	100	*S. intermedius*	Exc (4)	
			S. intermedius or *S. constellatus* subsp. *pharyngis*	LD (1)	
			S. constellatus subsp. *pharyngis*	Good (1)	
3	*S. constellatus*	100	Inconclusive identification	— (2)	2
			S. pyogenes, ***S. constellatus*** subsp. ***constellatus***, or *S. dysgalactiae* subsp. *equisimilis*	LD (1)	
2	*S. constellatus* or *S. intermedius*	96.9	*S. constellatus* subsp. *constellatus*	Exc (1)	
			S. constellatus subsp. ***constellatus***, *S. gordonii*, or ***S. anginosus***	LD (1)	1
S. sanguinis group					
7	*S. sanguinis*	100	*S. sanguinis*	Exc (6) or Good (1)	
2	*S. sanguinis*	96.9	*S. sanguinis*	Exc (2)	
5	*S. sanguinis*	93.7	*S. mitis*/*S. oralis*	Exc (2), VG (1), or Good (1)	4
			S. mitis/***S. oralis*** or *S. constellatus* subsp. *pharyngis*	LD (1)	1
4	*S. sanguinis*	90.5	*S. mitis*/*S. oralis*	Exc (2), VG (1), or Good (1)	4
1	*S. gordonii*	100	*S. gordonii*	Exc (1)	
1	*S. gordonii*	87.4	*S. gordonii*	Exc (1)	
S. salivarius group					
5	*S. salivarius* or *S. vestibularis*	100	*S. salivarius*	Exc (3)	
			S. infantarius	VG (1)	1
			Unidentified organism	— (1)	1
2	*S. salivarius* or *S. vestibularis*	96.9	*S. salivarius*	Exc (2)	
S. mitis group					
4	*S. mitis*	100	*S. mitis*/*S. oralis*	Exc (1), VG (1), Good (1), or Acc (1)	
1	*S. oralis*	100	*S. mitis*/*S. oralis*	Exc (1)	
24	*S. mitis, S. pneumoniae*, or *S. pseudopneumoniae*	96.9	*S. mitis*/*S. oralis*	Exc (10), VG (5), or Good (3)	
			S. pluranimalium or *S. mitis*/*S. oralis*	LD (1)	1
			S. mitis/***S. oralis*** or *S. constellatus* susbp. *pharyngis*	LD (1)	
			Unidentified organism	— (4)	4
6	*S. infantis*	93.7	*S. mitis*/*S. oralis*	Exc (2), VG (2), or Good (1)	
			Unidentified organism	— (1)	1
1	*S. oralis*	93.7	*S. mitis*/*S. oralis*	VG (1)	
1	*S. oralis*	90.5	*S. mitis*/*S. oralis*	Exc (1)	
1	*S. peroris*	93.7	*S. mitis*/*S. oralis*	Exc (1)	
3	*S. mitis* or *S. oralis* (v2)[e]	100	*S. mitis*/*S. oralis*	Exc (1) VG (1), Good (1)	
S. bovis group					
2	*S. gallolyticus* subsp. *gallolyticus*	100	*S. gallolyticus*	Exc (2)	
2	*S. bovis* or *S. lutetiensis*	100	*S. infantarius*	VG (1), Exc (1)	
1	*S. gallolyticus* subsp. *pasteurinanus*	100	*S. pasteurianus*	Exc (1)	
2	*S. bovis* or *S. lutetiensis*	96.9	*S. infantarius*	Exc (1)	
			S. gallolyticus, S. lutetiensis/bovis or S. hyointestinalis	LD (1)	
Undefined group					
2	*S. oralis* or *S. gallinaceus* (v1)[e]	100	*S. parasanguinis*	VG (1)	1
			S. parasanguinis or *S. mitis*/*S. oralis*	LD (1)	1
1	Both regions heterogeneous[f]		*S. mitis*/*S. oralis*	Exc (1)	1

[a] The isolates have been listed according to the pyrosequencing result.

[b] The score is a measure of the relatedness of the studied sequence to the type strain sequence determined by the Identifire software. Score 100 is given to sequences that are identical to each other.

[c] Confidence level is a measure of the strength of the VITEK analysis in the descending order Exc (excellent), VG (very good), good, Acc (acceptable), and LD (low discrimination). If VITEK 2 analysis resulted in low discrimination between two species and an additional Voges-Proskauer test proposed by the device could discriminate the species, the final result is in boldface.

[d] The number of isolates having discrepant pyrosequencing and VITEK 2 results at the group level.

[e] The isolates with a heterogeneous sequence in either region were identified using the other region.

[f] One isolate had heterogeneous sequences in both regions, and hence it could be identified only as *Streptococcus* by pyrosequencing.

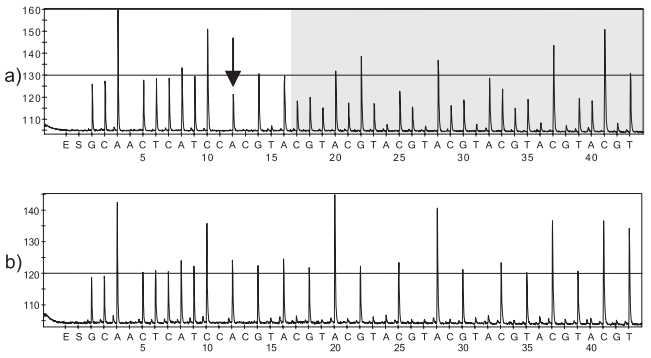

FIG. 1. Pyrogram of the v1 region obtained with a blood culture isolate carrying apparently at least two different v1 sequences. (a) The sequence is unambiguous (GCAACTCATCC) until the 12th dispensation, where apparently only some of the alleles contain the A nucleotide. After the 16th dispensation, interpretation of this pyrogram is hard. (b) Pneumococcal sequence using the same dispensation order. The height of the peaks indicating single nucleotide incorporations remain at approximately the same level in this pyrogram, indicating homogeneous 16S rRNA alleles. The pneumococcal v1 sequence is GCAACTCATCCAGAGAAGCAAGCTCCTCCTT.

ascertained to be *Streptococcus* also by pyrosequencing as the sequences were readable at least 10 bases from the beginning of the sequencing reaction.

Excluding the strains with a pyrosequencing score of <96.9% for all type strains, VITEK 2 and pyrosequencing achieved over 85% congruence in all streptococcal groups. One strain with v1 and v2 sequences identical to the *S. salivarius* type strain was identified as *S. infantarius*, which may be included in either the *S. bovis* or the *S. salivarius* groups according to its bile esculin reaction (8). Only three isolates with sequences identical to those of *S. gallolyticus* species were included, and they were correctly identified by VITEK 2.

Identification of streptococcal pharyngeal isolates. Of the 34 pharyngeal isolates, 4 (11.8%) could be unambiguously identified by pyrosequencing (Table 3), 2 could be identified as *S. mitis*, 1 could be identified as *S. parasanguinis*, and 1 could be identified as *S. salivarius* or *S. vestibularis*. Of these, only the *S. parasanguinis* isolate was viable for the VITEK analysis and

TABLE 3. Pyrosequencing and VITEK 2 results of the pharyngeal normal microbiota isolates

Group and no. of strains	Pyrosequencing result[a]		VITEK 2 result	
	Species	Score[b]	Species	Confidence level[c] (*n*)
S. mitis group				
2	*S. mitis*	100[d]	ND[e]	— (2)
4	*S. mitis*	96.9	*S. intermedius*	Exc (1)
			S. mitis/*S. oralis*	Exc (1) or VG (2)
1	*S. infantis*	96.9	ND	— (1)
1	*S. pneumoniae, S. pseudopneumoniae*, or *S. mitis*	96.9	ND	— (1)
1	*S. cristatus, S. infantis* or *S. peroris*	93.7	*S. mitis*/*S. oralis*	Exc (1)
1	*S. infantis*	93.7	*S. mitis*/*S. oralis*	Exc (1)
1	*S. pneumoniae, S. pseudopneumoniae* or *S. mitis*	93.7[d]	ND	— (1)
1	*S. oralis*	90.5	*S. mitis*/*S. oralis*	VG (1)
1	*S. infantis*	84.2[d]	*S. parasanguinis*	VG (1)
1	*S. australis*	87.4[d]	ND	— (1)
1	*S. mitis* or *S. oralis* (v2)	100[f]	*S. salivarius*	Exc (1)
1	*S. australis* (v2)	100[d,f]	*S. constellatus* subsp. *pharyngis*	Good (1)
2	*S. mitis, S. oralis*, or *S. infantis* (v2)	93.7[d,f]	*S. mitis*/*S. oralis*	VG (1) or Good (1)
S. sanguinis group				
1	*S. parasanguinis*	100	*S. parasanguinis*	Exc (1)
2	*S. gordonii*	96.9	*S. gordonii*	Exc (1)
			ND	— (1)
1	*S. sanguinis*	96.9	ND	— (1)
1	*S. sanguinis*	90.5	*S. mitis*/*S. oralis*	Acc (1)
5	*S. parasanguinis*	93.7	*S. parasanguinis*	VG (2) or Exc (3)
1	*S. parasanguinis*	90.5[d]	Inconclusive identification	— (1)
2	*S. parasanguinis* (v2)	93.7[d,f]	*S. parasanguinis*	Exc (1) or Good (1)
S. salivarius group				
1	*S. salivarius* or *S. vestibularis*	100	ND	— (1)
1	*S. salivarius* or *S. vestibularis*	96.9[d]	*S. salivarius*	Acc (1)
Undefined group				
1	*S. gallinaceus*	90.5	*S. mitis*/*S. oralis*	Exc (1)

[a] The isolates have been listed according to the pyrosequencing result.
[b] The score is a measure of the relatedness of the studied sequence to the type strain sequence determined by the Identifire software. Score 100 is given to sequences that are identical to each other.
[c] Confidence level is a measure of the strength of the VITEK analysis in the descending order Exc (excellent), VG (very good), good, Acc (acceptable), and LD (low discrimination).
[d] One of the isolates with this pyrosequencing result was sequenced by conventional sequencing. The GenBank accession numbers of the sequences are EF151144 to EF151152.
[e] ND, not determined.
[f] The pyrograms of the v1 region of these isolates indicated heterogeneous 16S rDNA alleles, and the isolates were tentatively identified using the v2 region.

TABLE 4. Agreement between the identifications of pyrosequencing and VITEK 2

Pyrosequencing score (*n*)	VITEK confidence level[a]	No. (%) concordant		No. (%) discordant
		Species level	Group level	
≥96.9 to one species (43)	Exc or VG	29		1
	Good or Acc	3	1	
	LD (resolved)	5		
	LD (unresolved)	0	1	
	Unidentified	0		3
	Subtotal	37 (86.0)	2 (4.65)	4 (9.30)
≥96.9 to two or three species (38)	Exc or VG	5	19	1
	Good or Acc	1	3	
	LD (resolved)		2	
	LD (unresolved)			2
	Unidentified			5
	Subtotal	6 (15.8)	24 (63.2)	8 (21.1)
<96.9 to all species (43)	Exc or VG	12	8	11
	Good or Acc	2	2	4
	LD (unresolved)			1
	LD (resolved)			1
	Unidentified			2
	Subtotal	14 (32.6)	10 (23.3)	19 (44.2)
All isolates (124)	Total	57 (46.0)	36 (29.0)	31 (25.0)

[a] Confidence level is a measure of the strength of the VITEK analysis in the descending order Exc (excellent), VG (very good), good, Acc (acceptable), and LD (low discrimination).

was identified as *S. parasanguinis* also by VITEK. Of the 18 isolates with one or two mismatches to the closest type strain by pyrosequencing, 13 were viable for the VITEK analysis. These isolates were assigned to the *S. mitis* ($n = 6$), *S. sanguinis* ($n = 6$), or *S. salivarius* ($n = 1$) group by pyrosequencing and were identified to the same streptococcal group with VITEK except for one isolate. Five isolates with lower Identifire scores were variably identified by using VITEK as well. Six pharyngeal isolates had apparently heterogeneous 16S rDNA alleles in the v1 region (Fig. 1 and Table 3), and four of them were subcultured several times in order to purify these potentially mixed isolates. However, the pyrograms of these isolates remained changeless after the subculturing.

Overall agreement of the results. Ninety-three (75.0%) of the isolates were identified at least to the same streptococcal group by both methods, and fifty-seven (46.0%) reached consistent results at the species level (Table 4). Of the 31 isolates with inconsistent results, 12 (36.4%) had low pyrosequencing scores (<96.9) or had heterogeneous sequences ($n = 4$ [12.1%]) and 11 (34.4%) isolates could not be conclusively identified by VITEK. In addition, two isolates could not be reliably identified by either method, and two isolates had discrepant results, although both results seemed to be of high confidence. Importantly, the confidence levels or pyrosequencing scores of the incongruent results were often low (Tables 2 to 4), whereas 48 of the 62 (77.4%) VITEK results with excellent confidence levels were congruent with pyrosequencing results at the species level when *S. mitis*/*oralis* was considered as a counterpart of the unresolved pyrosequencing result *S. pseudopneumoniae*/*S. pneumoniae*/*S. mitis*. (Tables 2 to 3).

DISCUSSION

The taxonomy of especially alpha-hemolytic streptococci is complex, and new species are constantly described (8). Furthermore, a proper gold standard for the identification of streptococci is not available, and therefore we had to compare the pyrosequencing and VITEK 2 results to each other in the present study. The results of the present study illustrate the difficulty of identifying alpha-hemolytic streptococci and the poor knowledge of streptococci of the human normal microbiota. However, the identification of alpha-hemolytic streptococci is becoming more and more important as the amount of immunocompromised patients is increasing. In addition to discrimination between *S. pneumoniae* and other species of the *S. mitis* group, the frequent isolation of *S. mitis* group members from patients with hematologic malignancies and the increasing penicillin resistance of these strains may make their identification a relevant issue (7, 16). Species of the *S. anginosus* group may have differing clinical importance (4), and high accuracy in the identification of *S. bovis* group isolates would be important with regard to the association between the isolation of *S. gallolyticus* subsp. *gallolyticus* or *S. gallolyticus* subsp. *pasteurianus* from blood culture and malignant or premalignant colon lesions (29, 31). However, group-level identification of alpha-hemolytic streptococci may be sufficient for clinical purposes in most cases.

Since the majority of the variation between the 16S rRNA gene sequences of streptococcal species is located close to the 5′ end of the gene, the discriminatory power of the v1 and the v2 region is close to that of the full-length 16S rRNA gene sequence. This was clearly seen also in the present study since the database search results with the longer sequences

were in agreement with the pyrosequencing results. Furthermore, the 30-nucleotide sequences of the v1 and v2 regions differentiated the type strains quite well, since only very closely related species belonging to the *S. salivarius* or *S. bovis* group had identical v1+v2 sequences. The difficulty in differentiating these species is not surprising since they have highly homologous 16S rRNA genes, as well as *rnpB*, *rpoB*, and *tuf* sequences (2, 6, 22, 23, 32, 37). On the other hand, the *rnpB* pyrosequencing method could not differentiate *S. anginosus* and *S. constellatus* (15).

In the first published evaluation of the colorimetric GP card, all of the 18 alpha-hemolytic isolates were correctly identified (9). Innings et al. (15) compared pyrosequencing of the *rnpB* gene and VITEK 2 with the fluorimetric GPC card in the identification of 113 blood culture isolates, 44 of which were nonpneumococcal alpha-hemolytic streptococcal isolates. In that study, 36 (81.8%) of the 44 isolates could be unambiguously identified by the pyrosequencing method, and the pyrosequencing and VITEK results of 34 (77.3%) isolates were concordant at least at the group level. Of the 10 isolates with nonconformant results, 9 (90.0%) were isolates that could not be identified by using VITEK. In the present study, the pyrosequencing and VITEK 2 results of 124 streptococcal isolates were compared, and 93 (75.0%) isolates reached concordant results at least at the group level. Most of the discrepant results were found in the *S. mitis* and *S. sanguinis* groups, and many of the isolates had low pyrosequencing scores, indicating variation from the type strain. The species belonging to the *S. mitis* and *S. sanguinis* groups are difficult to differentiate, and they are often regarded as a single group (30). In contrast, 30 (83.3%) of the 36 isolates belonging to other groups by both methods were concordantly identified, and five of the six discordant results were due to the low quality of the VITEK result. Consequently, the quality controls of both methods seem to be quite reliable, and the results with lower quality can be used for group-level identifications at best.

Most of the sequences obtained in the present study were available in GenBank, but since the species epithets of other than type strains may be incorrect, only the streptococcal type strains can be reliably used for species identification (14). The higher rate of low pyrosequencing scores in our study compared to the study of Innings et al. is also most likely due to the fact that only type strain sequences were included in our sequence database, whereas other reference strains were also included in the database of Innings et al. This illustrates the need for careful study of streptococci to determine the amount of intraspecies sequence variation, which is almost impossible before the species designations of reference strains are ascertained (19). On the other hand, since the smallest difference between the combined v1 and v2 sequences of several streptococcal species belonging to the *S. bovis* group was only one nucleotide, there may be members of yet-undescribed species among the isolates of the present study. The high degree of variation in streptococcal 16S rRNA gene sequences detected in the present study also indicates that bacteria have a continuum of characters. Consequently, the 16S rRNA gene is unable to give every isolate a clear definition, and the same applies to any species definition.

The pyrosequencing method used in the present study is reliable, user-friendly, and rapid: the whole procedure from a streptococcal colony on an agar plate to the 16S rRNA gene sequence can be carried out during a single working day. Very similar results were obtained with this method compared to the previously published *rnpB* pyrosequencing method (15). One advantage of the present method is the ability of the primers to also amplify other than streptococcal DNA, whereas the *rnpB* method failed to amplify the DNA of eight streptococcal species (15). Therefore, presumptive identification before pyrosequencing analysis by this method is sufficient. The pyrosequencing method could also be useful in clinical microbiology laboratory since the major pathogens had specific v1 and v2 sequence combinations. Most importantly, *S. pneumoniae* had a specific sequence, and no intraspecies variation could be found among the studied pneumococcal isolates or among available genomic sequences.

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